INHIBITION OF Ca^{2+} TRANSPORT IN MITOCHONDRIA BY SELECTIVE BLOCKADE OF MEMBRANE MUCOPOLYSACCHARIDES BY HEXAMINE COBALTICHLORIDE

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1. Introduction

It has recently been found that the specific dye ruthenium red, widely used in cytochemical investigations for identifying mucopolysaccharides, effectively inhibits Ca^{2+} transport in mitochondria with practically no effect at the same concentrations on the energy transformation process [1, 2]. According to the literature [3, 4] glycoproteids are found in both outer and inner mitochondrial membranes. Apparently some of the inner membrane glycoproteids may be involved in the bivalent ion transporting system [1].

In order to elucidate the intermediate steps of calcium accumulation and determine the role of the functional groups in the mucopolysaccharide-containing components of the membrane the effect of hexamine cobaltichloride, $[Co(NH_3)_6]Cl_3$ (HAC), upon calcium transport and certain other functional parameters of intact rat liver mitochondria and upon the calcium binding by the acid glycoproteid isolated from the inner mitochondrial membrane has been investigated. This complex cation reacts with the negatively charged sites of sulfate-containing glycosamineglycans and is used for determining the total amount of their anionic groups [5].

2. Materials and methods

Rat liver mitochondria were isolated and washed in medium containing 250 mM sucrose and 5 mM Tris-HCl (pH 7.4) according to the common procedure. The kinetics of calcium transport and ATP synthesis in intact mitochondria were determined by recording the pH changes of the suspensions. Other functional parameters of the mitochondria were measured by polarographic and fluorimetric methods. The protein content was determined by the biuret reaction. The "ghosts" of inner mitochondrial membranes were prepared mainly by Lehninger's method [6], 5 mM Tris-HCl (pH 7.4) being used for the osmotic shock. Acetone powders of the mitochondrial "ghosts" were prepared by Selwyn's method as described for intact mitochondria [7].

An aqueous extract of glycoproteid was obtained by suspending the acetone powder in a glass homogeniser (80 mg powder per 1 ml of water). After extraction with continuous stirring for 1 hr the suspension was centrifuged at 22,000 *g* for 30 min. The supernatant was run through a G-50 Sephadex column and eluted with 2 mM Tris-HCl solution (pH 7.4). The second fraction, containing the glycoproteid, was collected and concentrated on a rotatory evaporator. The calcium binding activity of the fraction was determined by passing it through a G-50 Sephadex column in the presence of 6×10^{-6} M $[^{45}Ca]CaCl_2$ containing various amounts of HAC. The eluate fractions were measured for their protein content at 280 nm and for the ^{45}Ca radioactivity level.

Disc-electrophoresis was carried out in 7% polyacrylamide gel at pH 8.9. Proteins were determined by staining with amido-black and mucopolysaccharides by means of toluidine blue (0.1% solution in acetate buffer pH 4.5).

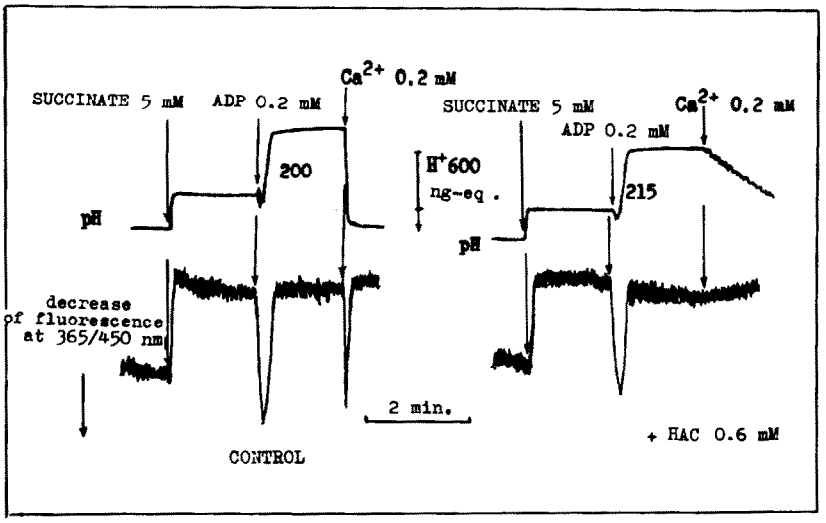

Fig. 1. Effect of HAC on the Ca^{2+}- and ADP-induced changes of the redox-state of endogenous NAD(P) and on the H^+ movement in a rat liver mitochondria suspension. Incubation medium contained 120 mM KCl, 5 mM Tris-HCl, 2.5 mM KH_2PO_4; pH = 7.4. Room temperature, sample volume 6 ml. The phosphorylation rate is designated on the pH curve.

3. Results and discussion

The study of the action of HAC on functional parameters of energized mitochondria has shown that this complex cation specifically inhibits calcium transport and that it has no effect whatsoever on other endergonic processes in the mitochondria. The effect of ADP and Ca^{2+} on the redox-state of mitochondrial pyridinenucleotides and the metabolically-induced pH shift of the suspension are represented in fig. 1. The decrease in rate of the functional responses of mitochondria on addition of calcium in the presence of HAC indicates inhibition of calcium translocation whereas the integrity of the energy transformation system is determined by complete retention of the response to the addition of ADP. The inhibitory effect of HAC on calcium transport increases together with increases in concentration of this complex (fig. 2), half-maximum inhibition being observed at 3×10^{-5} M HAC. At the same time, HAC in such concentrations has no effect on the mitochondrial respiration in metabolic states 3 and 4 (V_3 and V_4), on the phosphorylation rate (V_p), on the ADP/O ratio and on the respiratory control index (RC) (table). The only

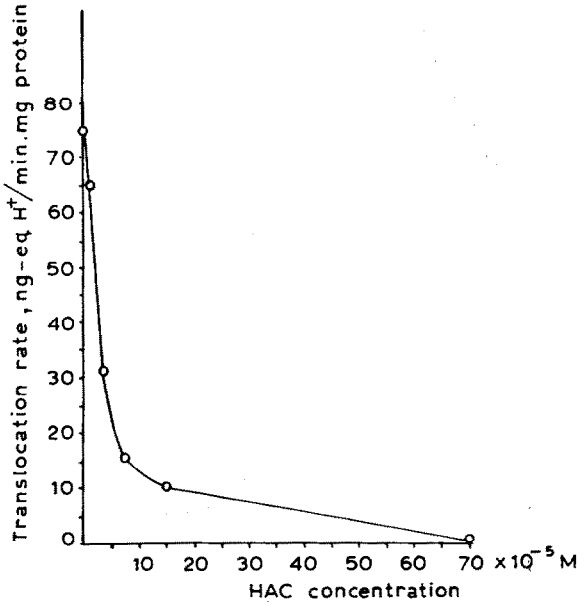

Fig. 2. Effect of various HAC concentrations on the calcium translocation rate in mitochondria. Incubation medium contains 120 mM KCl, 5 mM Tris-HCl, pH = 7.4, 3 mM succinate; room temp.

Table 1
Effect of different HAC concentrations on certain functional parameters of rat liver mitochondria.

	HAC concentration (μM)	V_3	V_4	V_p	RC	ADP/O	V_{Ca}
1.	0	102	26	154	4.0	1.6	88
2.	3	98	24	160	4.0	1.6	66
3.	12	96	25	152	4.0	1.6	57
4.	60	89	23	145	3.9	1.5	31
5.	90	92	22	152	4.1	1.5	24
6.	120	89	23	152	3.9	1.7	15

(Incubation medium contained 120 mM KCl, 10 mM Tris-HCl, 10 mM succinate, 2.5 mM KH_2PO_4; pH 7.4, volume of polarographic cell was 1 ml, temperature 25°. A stationary platinum electrode with magnetic stirring of the medium was used. Respiration rate in metabolic states V_3, V_4 and V_{Ca} is expressed in ng-atoms O_2/min per 1 mg of protein. V_{Ca} is the initial respiration rate after addition 200 ng-ions of Ca^{2+} minus the respiration rate in state V_4. V_p is the phosphorylation rate, in nmoles ADP/min per 1 mg of protein).

HAC-sensitive parameter is the rate $V_{Ca^{2+}}$ of the Ca^{2+} translocation-induced respiration. The inhibitory effect of HAC on calcium transport, contrary to that for the lanthanides, does not depend on the presence of phosphate in the medium. Therefore HAC can be considered as a more specific instrument for investigation of calcium transport and its coupling with the energy transfer systems in mitochondria. Since HAC is a selective reagent for the anionic groups of mucopolysaccharides its effect on intact mitochondria may be due to its interaction with similar groups taking part in the transport of bivalent cations in the mitochondria.

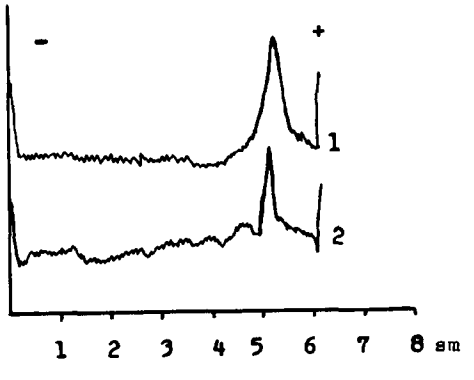

Fig. 3. Electrophoretic patterns of the mitochondrial glycoproteid stained with toluidine blue (1) and with amido-black (2).

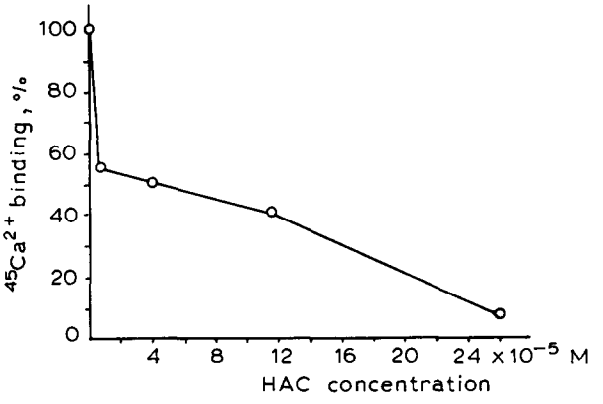

Fig. 4. Effect of HAC on ^{45}Ca binding by mitochondrial glycoproteid. Calcium binding in absence of HAC was considered as 100%.

An acid glycoproteid isolated from the inner mitochondrial membrane (fig. 3) has a molecular weight of about 5,000 by Andrews's method [8]. This component of the mitochondrial membrane effectively binds calcium, the process diminishing in the presence of HAC depending upon the concentration used (fig. 4). Moreover, it was found that approximately the same concentration of HAC induces half-maximum inhibition of binding $^{45}Ca^{2+}$ by the glycoproteid and lowers by half the rate of calcium transport in the intact mitochondria.

The experiments described above with HAC show that the anionic groups of specific glycoproteins of the inner membrane are effective binding centres in the calcium transporting system of mitochondria.

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